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(54) Title: RECOMBINANT XYLANASE

(57) Abstract

A method of cloning of xylanase clones from an anaerobic rumen fungus including the steps of: (i) cultivation of an anaerobic rumen fungus; (ii) isolating total RNA from the culture in step (i); (iii) isolating poly A+ mRNA from the total RNA referred to in step (ii); (iv) constructing a cDNA expression library; (v) ligating cDNA to a bacteriophage expression vector selected from λZAP, λZAPII or vectors of similar properties; (vi) screening of xylanase positive recombinant clones in a culture medium incorporating xylan by detection of xylan hydrolysis; and (vii) purifying xylanase positive recombinant clones. There is also provided xylanase positive recombinant clones produced by the above-mentioned method as well as xylanase positive recombinant clones having the following properties: (i) production of xylan clearing zones in a culture containing xylanase cDNA derived from *N. patriciarum*; (ii) having activity in hydrolysis of xylan but having no activity in relation to hydrolysis of CMC or crystalline cellulose. There is also provided various cDNA molecules which may be utilised in the above-mentioned method.

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TITLE

"RECOMBINANT XYLANASE"

FIELD OF INVENTION

This invention relates to a recombinant xylanase derived from an anaerobic fungus and a method of production of the recombinant xylanase and clones utilised in the method.

BACKGROUND ART

Xylan is a major component of hemicellulose and the second major component of plant fibre. Xylan consists of a backbone of β-1,4-linked xylose units. The enzymic cleavage of ß-1,4-xylosidic linkages is performed by endo-ß-1,4-xylanases (xylanases). Many microorganisms produce extracellular xylanases. In the past decade, many xylanase genes were isolated from lignocellulolytic bacteria, but isolation f xylanase genes from fungi with functional expression in E. coli has not been documented prior to this invention.

Lignocellulolytic fungi usually produce more active xylanase than bacteria, in particular, the anaerobic fungus *Neocallimastix patriciarum*, isolated from the sheep rumen, has a high capacity for xylan degradation.

Reference may also be made to other prior art which serves as background prior art prior to the advent of the present invention. Such prior art includes:

(i) Reymond et. al. Gene 110 (1992) 57-63;

(ii) Wong et. al. Clin. Reviews in Biotechnology 12 413-435 (1992);

(iii) Orpin et. al. Current Microbiology Vol 3 (1979) pp 121-124;

(iv) Mountfort and Asher in "The Roles of Protozoa and Fungi in Ruminant Digestion" (1989) Pernambul Books (Australia);

(v) Joblin et. al. FEMS Microbiology Letters 65 (1989) 119-122;

(vi) Lowe et. al. Applied and Environmental Microbiology June 1987 pp 1210-1215; and

(vii) Lowe et. al. Applied and Environmental Microbiology June 1987 pp 1216-1223.

Cloning of xylanase genes from bacteria can be achieved by isolation of enzymatically active clones from genomic libraries established in *E. coli*. However this approach for isolation of xylanase genes from fungal genomic libraries with functional expression of xylanase is not possible. This is because fungi are eucaryotic microorganisms. Most eucaryotic genes contain introns and *E. coli* is unable to perform post-transcriptional modification of mRNAs in order to splice out introns. Therefore, enzymatically functional protein cannot normally be synthesised in clones obtained from a fungal genomic library.

The cDNA cloning approach can be used to overcome the post-transcriptional modification problem in *E. coli*. However, xylanases in fungi are usually glycosylated and glycosylation is often required for biological activity of many glycosylated enzymes. E. coli lacks a glycosylation mechanism. This problem can be solved if the cloned gene is transferred to an eucaryotic organism, such as yeast. Other problems which are often encountered in obtaining a biologically functional protein from a cDNA clone in *E. coli* are (i) that many eucaryotic mRNAs contain translational stop codons upstream of the translational start codon of a gene which prevents the synthesis of the cloned protein from the translational start provided in the vector, and (ii) that synthesis of the cloned protein is based on fusion proteins and the biological function of the cloned protein is often adversely affected by the fused peptide derived from the cloning vector.

Therefore, in the past, researchers in this field employed differential or cross hybridisation, antibody probes or oligonucleotide probes for the isolation of fungal polysaccharide hydrolase cDNA or genomic DNA clones. Relevant publications in this regard include

Reymond et. al. FEMS Microbiology letters 77 (1991) 107-112; Teeri et. al., Biotechnology 1 696-699 (1983); Shoemaker et. al., Biotechnology 1 691-696 (1983); Sims et. al. Gene 74 411-422 (1988); Morosoli and Durand FEMS Microbiology Letters 51 217-224 (1988); and Azevedo et. al. in J. Gen. Microbiol. 136 2569-2576 (1990). However, these methods are very time-consuming, and quite often two stages of intensive cloning work are required for isolation of an enzymatically functional clone. For antibody or oligonucleotide probes, purification of the fungal xylanase is also required. It usually takes more than one year to obtain a functional enzyme clone using the above approaches.

Isolation of fungal xylanase cDNAs by utilising an expression system in E. coli has not been reported prior to the advent of this invention probably at least partially due to failure in obtaining enzymatically functional xylanase clones by using improper expression vectors. Selection of expression vector systems is important. If plasmid expression vectors such as pUC vectors are used, and the cloned enzyme is trapped inside the cell, therefore screening for xylanase clones by the convenient xylan-agar plate technique is difficult. Bacteriophage vectors have an advantage in respect to the release of the cloned enzyme into xylan-agar medium due to cell lysis. However, commonly used bacteriophage expression vectors, λgt11 and its derivatives, have polyclonal sites at the C-terminus of the LacZ peptide. The large part of LacZ peptide fused to the cloned enzyme often adversely affects the cloned enzyme activity.

In specific regard to the abovementioned Reymond et. al. (1991) reference there is described an attempt of molecular cloning of polysaccharide hydrolase (ie. cellulase) genes from an anaerobic fungus which is N. frontalis. In this reference a clone from a cDNA library derived from N. frontalis hybridized to a DNA probe encoding part f the exo-cellobiohydrolase (CBH 1) gene of Trichoderma rees i. However it was subsequently r vealed by Reymond et. al. in a personal

communication that the particular cDNA clone obtained from N. frontalis does not encode any polysaccharide hydrolase.

Moreover the Reymond et. al. reference did not describe the production of biologically functional enzymes from these clones.

In relation to isolation of a fungal xylanase gene, the only report that exists so far prior to this invention is the abovementioned Morosoli and Durand reference which describes isolation of a xylanase gene from yeast Cryptococcus albidus using differential hybridization techniques. However, this reference does not describe the production of biologically functional enzymes from this xylanase gene.

BROAD STATEMENT OF INVENTION

It is an object of the invention to provide a recombinant xylanase from an anaerobic rumen fungus which may be of use commercially in relation to hydrolysis of xylan.

A further object of the invention is to provide a method of cloning of xylanase cDNAs from an anaerobic rumen fungus which may encode the recombinant xylanase of the invention.

A further object of the invention is to provide xylanase clones which may be produced in the abovementioned method.

The method of cloning of the invention includes the following steps:

(i) cultivation of an anaerobic rumen fungus;

(ii) isolating total RNA from the culture in step (i);

(iii) isolating poly A^+ mRNA from the total RNA referred to in step (ii);

(iv) constructing a cDNA expression library;

(v) ligating cDNAs to a bacteriophage expression vector selected from λZAP, λZAP II or vectors of similar properties;

(vi) screening of xylanase positive recombinant clones in a culture medium incorporating xylan by detection of xylan hydrolysis; and

(vii) purifying xylanase positive recombinant clones.

In step (i) above in relation to preparation of the recombinant xylanase, from anaerobic fungi, particularly alimentary tract fungi, may be cultivated as described hereinbelow. These fungi are strict anaerobes and may be exemplified by *Neocallimastix patriciarum, Neocallimastix frontalis, Neocallimastix hurleyensis, Neocallimastix stanthorpensis, Sphaeromonas communis, Caecomyces equi, Piromyces communis, Piromyces equi, Piromyces dumbonica, Piromyces lethargicus, Piromyces mai, Ruminomyces elegans, Anaeromyces mucronatus, Orpinomyces bovis and Orpinomyces joyonii.* In regard to the above mentioned anaerobic alimentary tract fungi, *Caecomyces equi, Piromyces equi, Piromyces dumbonica and Piromyces mai* are found in horses and thus are not located in the rumen of cattle like the other fungi described above.

The cultivation may proceed in appropriate culture media containing rumen fluid and also may contain cellulose such as Avicel (ie. a form of microcrystalline cellulose) as a carbon source under anaerobic conditions. After cultivation of the fungi total RNA may be obtained in any suitable manner. Thus initially the fungal cells may be harvested by filtration and subsequently lysed in appropriate cell lysis buffer by mechanical disruption. A suitable RNA preserving compound may also be added to the fungal cells to maintain the RNA intact by denaturing RNAses which would otherwise attack the fungal RNA. The total RNA may subsequently be isolated from the homogenate by any suitable technique such as by ultracentrifugation through a $CsCl_2$ cushion or alternative technique as described by Sambrook et. al. in Molecular Cloning; A Laboratory Manual 2nd Edition Cold Spring Harbor Laboratory Press in 1989. An alternative method for preparation of total fungal RNA to that described above may be based on or adapted from the procedure described in Puissant and Houdebine in Bio-Techniques 148-149 in 1990. Total fungal RNA in this alternative technique may also be isolated from the above homogenate by extraction with phenol

chloroform at pH4 to remove DNA and associated protein. Total RNA obtained was further purified by washing with lithium chloride-urea solution.

Poly $(A)^+$ mRNA may then be isolated from the total RNA by affinity chromatography on a compound containing multiple thymine residues such as oligo (dT) cellulose. Alternatively a compound containing multiple uracil residues may be used such as poly (U)-Sephadex. The poly $(A)^+$ mRNA may then be eluted from the affinity column by a suitable buffer.

A cDNA expression library may then be constructed using a standard technique based on conversion of the poly $(A)^+$ mRNA to cDNA by the enzyme reverse transcriptase. The first strand of cDNA may be synthesised using reverse transcriptase and the second strand of the cDNA may be synthesised using *E. coli* DNA polymerase I. The cDNA may subsequently be fractionated to a suitable size and may be ligated to the bacteriophage expression vector, preferably λZAP or λZAPII. The cDNA library may then be amplified after packaging *in vitro*, using any suitable host bacterial cell such as a suitable strain of *E. coli*.

The choice of the bacteriophage expression vector in step (v) is important in that such expression vector should include the following features:

(i) having an E. coli promoter;

(ii) having a translation start codon;

(iii) having a ribosomal binding site;

(iv) the fusion peptide derived from the vector should be as small as possible as the biological function of the cloned protein is usually adversely affected by the fused peptide derived from the vector. Therefore the polyclonal sites of the bacteriophage expression vector are suitably located at the N-terminus of lacZ peptides such as in λZAPII.

It will be appreciated from the foregoing that if an expression vector is utilis d as described above the chances of obtaining a

biologically functional enzyme is greatly increased. Isolation of many enzymatically functional xylanase clones in the present invention as described hereinafter has proved the efficiency of this approach. To our knowledge this is the first record of isolation of xylanase cDNA clones with functional enzyme activity from anaerobic fungi based upon the expression of recombinant bacteriophage in E. coli using an expression vector such as that described above. λZAP and λZAP II are examples of such expression vectors.

Therefore the term "vectors of similar properties" to λZAP r λZAPII includes within its scope expression vectors having the abovementioned features (i), (ii), (iii) and (iv).

It is also clear from the product summary which accompanies the λZAPII vector as supplied by the manufacturer that in relation to fusion protein expression that such fusion proteins may only be screened with antibody probes. Clearly there was no contemplation that the λZAPII vector could be utilised for screening of clones involving enzymic expression on a suitable substrate or any direct screening by biological activity. When it is realised that the present invention involves expression in a bacterial host cell such as E. coli of a cDNA of eucaryotic origin (ie. fungal origin) then the novelty of the present invention is emphasised.

The screening of xylanase positive recombinant clones may be carried out by any suitable technique based on hydrolysis of xylan. In this procedure the clones may be grown on culture media incorporating xylan and hydrolysis may be detected by the presence of xylanase-positive plaques suitably assisted by a suitable colour indicator. Xylanase positive recombinant clones may then be purified and the cDNA insert in the clones may then be excised into pBluescript (SK(-)) to provide an expression vector of simplified structure when compared to the λZAP II construct which will enhance expression of the xylanase in E. c li.

Any suitable E. coli promoter may be used in the expression vector described above. Suitable promoters include lacZ, Tac, Bacteriophage T_7 and lambda-P_L.

The recombinant xylanase enzyme may then be characterised and principal features that have been ascertained are as follows:

(i) the cloned xylanases have high specific activity.

(ii) the enzyme has no residual activity against cellulose, while many other xylanases possess some cellulase activity. This property of the xylanase is particularly useful in its application to pulp and paper industry to remove xylan and dissociate lignin from plant fibre without damaging cellulose fibre.

The high specific activity of the cloned xylanases is an excellent intrinsic property of this fungal xylanases. The expression level of the present constructs of xylanase cDNAs can be further improved by manipulating the gene and promoters.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Experimental Methods

1. Microbial strains, vectors and culture media.

The anaerobic fungus *Neocallimastix patriciarum* (type species) was isolated from a sheep rumen by Orpin and Munn (1986) in Trans. Br. Mycol. Soc. 86 178-181 and cultivated in the laboratory for many years under selection by lignocellulose substrates. Host strains for cDNA cloning and characterisation of the recombinant xylanases were *E. coli* PLK-F, XL1-Blue and JM83.

The vectors were λZAPII, pBluescript SK(-) (Stratagene). *N. patriciarum* culture was maintained in a medium containing 10% rumen fluid as described by Kemp et. al. (1984). *E. coli* strains were grown in L-broth as described by Sambrook et. al. (1989) for general purposes. The recombinant phage grown in *E. coli* strains using NZY medium according to Stratagene's instructions.

2. General recombinant DNA techniques.

Agarose-gel electrophoresis, transformation of *E. coli* and modification of DNA using restriction enzymes and T4 DNA ligase were as described in Sambrook et. al. above. The alkaline lysis method of Birnboim and Doly as described in Nucl. Acids Res. 7 1513-1523 (1976) as employed to isolate plasmid. In vitro DNA amplification by polymerase chain reaction (PCR) was based on the procedure described by Saiki (1989) in PCR Technology (H.A. Erlich, ed) pp. 7-16, M. Stockton Press, New York.

3. Cultivation of rumen anaerobic fungus, N. patriciarum for preparation of RNA.

N. patriciarum was grown in a rumen fluid-containing medium as described in Kemp et. al. J. Gen. Microbiol. 130 27-37 (1984) in the present of 1% Avicel at 39°C and under anaerobic conditions for 48hr (Alternative culture media, such as described by Philips and Gordon in Appln. Environ. Microbiol. 55 1695-1702 in 1989 and Lowe et. al. in J. Gen. Microbiol. 131 2225-2229 in 1985 can be used).

4 Total RNA isolation.

The frozen mycelia were ground to fine powder under liquid nitrogen with a mortar and pestle. 5-10 vol of guanidinium thiocyanate solution (4M guanidinium thiocyanate, 0.5% sodium laurylsarcosine, 25mM sodium citrate, pH7.0, 1mM EDTA and 0.1 M ß-mercaptoethanol) was added to the frozen mycelia powder and the mixture was homogenised for 5 min with a mortar and pestle and for further 2 min at full speed using a Polytron homogeniser. Total RNA was isolated from the homogenate by ultracentrifugation through a CsCl cushion (Sambrook et. al., 1989). (An alternative method for preparation of total fungal RNA, such as adaptation of the procedure described by Puissant and Houdebine in Bio-Techniques 148-149 in 1990 can be used).

5. Poly A^+ mRNA purification.

Poly A^+ was purified from the total RNA by Oligo (dT) cellulose chromatography (Sambrook et. al., 1989).

6. Construction of a cDNA expression library of *N. patriciarum*.

The cDNA library was constructed, using Stratagene's λZAP cDNA synthesis Kit, basically according to the manufacturer's instructions.

The procedure is described briefly as follows: PolyA$^+$, RNA was converted to the first strand cDNA by reverse transcriptase, using XhoI linker - oligo (dT) primer and 5-methyl dCTP. Double-stranded cDNA was synthesised from the first-strand cDNA by the action of RNase H and DNA polymerase I. After blunting cDNA ends, the cDNA was ligated with EcoR I adaptor, phosphorylated and digested with Xho1 to create cDNA with the EcoR I site at 5' region and the XhoI site at 3' region. The cDNA was size-fractionated by 1% low-melting point agarose gel electrophoresis and 1.2-8Kb sizes of the cDNA were recovered by phenol extraction (Sambrook et. at., 1989). The size-fractionated cDNA was then ligated to the EcoRI/XhoI digested λZAPII vector.

The cDNA library was packaged in vitro and amplified using *E. coli* PLK-F' as plating cells.

7. Screening xylanase-positive recombinant bacteriophage clones.

Recombinant phage were grown in *E. coli* XL1-Blue in 0.7% top agar containing 0.1% xylan and 10mM isopropyl-ß-thio-galactopyranoside (IPTG, an inducer for LacZ promoter controlled gene expression). After overnight incubation at 37°C, 0.5% Congo red solution was added over the top agar. After incubation at RT for 15 min, the unbound dye was removed by washing with 1 M NaCl. Xylanase-producing phage plaques were surrounded by yellow haloes against a red background.

The xylanase-positive recombinant phage were purified to homogeneity by replating and rescreening the phage as above for 2-3 times.

The cDNA insert in xylanase-positive phage were xcised into pBluescript SK (-) using R408 helper phage.

8. Xylanase and related-enzyme assays.

The cloned enzyme extracts from *E. coli* harbouring xylanase-positive recombinant plasmids were prepared by harvesting the cells by centrifugation. The cell pellet was suspended in 25mM Tris-Cl/ 2mM EDTA containing lysozyme (0.25mg/ml) and incubated on ice for 60 mins. After freezing, thawing and homogenisation, the crude cell lysate was used for enzyme assays.

The enzymes were assayed for hydrolysis of xylan or other substrates at 40°C in 50 mM Na-citrate, pH 6.5, except where otherwise indicated in the text. The reducing sugars released from xylan or other plant polysaccharides (Avicel) were measured as described by Lever in Anal. Biochem. 47 273-279 in 1972.

Xylanase activity on Kraft pulp was conducted as follows: Kraft pulp was suspended in tap water, and pH was adjusted to pH 7 with 1M H_2SO_4. The xylanase extract was added to the Kraft pulp suspension and the reducing sugar released was measured as above.

9. DNA sequencing.

Single-stranded plasmid DNA was prepared basically according to Stratagene's protocol. Sequencing of the resultant DNA was based on the protocol recommended by the manufacturer of the T7 DNA polymerase sequencing kit (Promega).

10. Optimisation of growth conditions of pNX-Tac clone.

E.coli strain JM83 harbouring pNX-Tac plasmid grew in LB/$Amp_{(100\mu g/ml)}$ at 30°C overnight. One millilitre of the overnight culture was inoculated into 100ml of media as specified in Table 5. IPTG was added at different times of growth. The cultures were grown at 30°C for 17hr, 24hr and 30hr. The cells were harvested for measurement of xylanase yield.

Results and discussion

Isolation and partial characterisation of xylanase cDNA clones.

A cDNA library consisting of 10^6 clones was constructed using mRNA isolated from *N. patriciarum* cells grown with Avicel as sole

carbon source. Thirty-one recombinant bacteriophage, which hydrolysed xylan, were identified after an initial screening of 5 x 10^4 clones from the library and 16 strongly xylanase-positive phage and two weakly xylanase-positive phage were isolated and purified. Xylanase activity of these recombinant bacteriophage clones was initially analysed by scoring xylan-hydrolysis zones (Fig. 1 and Table 1).

These 16 strongly xylanase positive clones were originally forwarded to Dr H J Gilbert and Dr G P Hazlewood of The University of Newcastle-upon-Tyne and the AFRC Institute of Animal Physiology and Genetics Research in the United Kingdom who carried out furth r analysis of these clones which included restriction mapping and hybridization analysis as well as sequencing of the longest clone. In this regard reference should be made to the publication "Homologous catalytic domains in a rumen fungal xylanase: evidence for gene duplication and prokaryolic origin" by H J Gilbert, G P Hazlewood, J I Laurie, C G Orpin and G P Xue which is published in Molecular Microbiology (1992) 6 (15) 2065-2072. The longest clone referred to in this reference is designated pNX1 and this corresponds to clone pNPX21 described hereinafter. In the Gilbert et. al. reference described above other plasmids pNX2, pNX3, pNX4, pNX5, pNX6 and pNX7 were produced as a result of truncation of pNX1 by restriction enzymes.

The clone corresponding to clone pNX1 in E. coli strain XL1-Blue described above has now been deposited at the International Depository ie. Australian Government Analytical Laboratories on June 22, 1992 under accession number N92/27542.

In an attempt to obtain more highly active xylanase clones, further screening of 4 x 10^5 clones from the library was conducted, which resulted in > 200 xylanase-positive clones. Ten highly active clones were isolated and purified. Two of these recombinant bacteriophage clones (λNPX29 and λNPX30) have much stronger xylanase activity than previously isolated high activity clones (see Table 1).

The cDNA inserts encoding *Neocallimastix patriciarum* xylanases were in vivo excised from bacteriophage (λZAP11) form into plasmid pBluescript SK⁻ form. Several clones with high xylanase activity were analysed for substrate specificity (four clones presented in Table 2). The xylanases produced by these clones have no activity on carboxymethyl-cellulose (CMC, a substrate for endo-glucanase) or Avicel (Avicel is crystalline cellulose and is a substrate for exo-glucanase). The restriction maps of the representative clones are presented in Fig. 2. It appears that these four xylanase cDNAs have the same restriction pattern but differ in length. pNPX13 and pNPX29 have shorter lengths than pNPX21 but they have much higher activity than pNPX21. Interestingly, pNPX30 has a similar length to pNPX21 but it has about 15-fold higher xylanase activity than pNPX21. Because of the remarkable difference in enzyme activity between pNPX21 and pNPX30, the xylanase cDNA of pNPX30 clone was sequenced. The result shows that DNA sequence of pNPX30 shares the same sequence with pNPX21 in a large part of cDNA, but differ in both the 5' and 3' regions. (Fig. 3). pNPX30 cDNA is not full-length. Interestingly, the N-terminus of pNPX30 xylanase has six repeated arginine/glutamic acid residues (Fig. 4).

The pH and temperature optima of xylanases produced by pNPX21 and pNPX30 were investigated. These enzymes were active in a wide range of pH and preferably at pH 5 - 8. The thermostability of these enzymes was tested at temperatures from 30°C - 60°C. The enzymes are active at 30°C- 55°C and preferably at 40°C - 50°C.

Genetic modification of *N. patriciarum* xylanase cDNA

pNPX30 (and pNPX21) contains two large repeated domains. Three main constructs were produced from pNPX30.

pNXD-Tac

pNPX30 plasmid (pNPX21 can also be used) was used as a template for in vitro DNA amplification by PCR for construction of pNXD-

Tac using primer I and primer IV (Fig.5). The amplified DNA was digested with EcoR1 and Hind111 and ligated to EcoR1 and Hind111 digested pBTac2 (Boehringer) to produce pNXD-Tac.

pNXS-Tac

pNXD-Tac plasmid was digested with Hind111 and blunted by filling-in with Klenow followed by partial digestion with Sca1. After fractionation on LMT agarose gel, the 5.3Kb band was recovered from the gel and ligated to produce the pDGXS construct, which has xylanase activity. pDGXS plasmid was used as a template for in vitro DNA amplification for construction of pNXS-Tac using primer I and primer II (Fig.5). The amplified DNA was digested with EcoR1 and Hind111 and ligated to EcoR1 and Hind111 digested pBTac2 vector to produce pNXS-Tac.

pNX-Tac

pNPX30 plasmid (pNPX21 or other xylanase cDNAs listed in Fig.2 can be used) was digested with RsaI and a 709bp fragment as indicated in Fig.5 was isolated after fractionation on agarose gel electrophoresis. The 709 fragment was ligated to Sma1 and Pst1 digested pUC18 (Pst1 end was blunted with T4 DNA polymerase). This construct is designated pNXP2 and the xylanase activity of this construct with the right orientation of truncated xylanase cDNA from pNPX30 confirmed that this fragment of the cDNA encodes a caterlytically functional domain. Two oligonucleotide primers, primer III and primer IV, (Fig.5) were then designed for PCR amplification of the pNXP2 xylanase cDNA insert. The PCR amplified fragment was digested with EcoR1 and Hind111 and ligated to EcoR1 and Hind111 digested pBTac2 vector to produce pNX-Tac.

These constructs are all modified at the N-terminal sequence of the truncated xylanase cDNA and a translational stop codon (TAA) was introduced into the end of the truncated xylanase coding region. The

expression of xylanase was controlled by the Tac promoter (Fig.6) and xylanases in these constructs are synthesised as nonfusion proteins. The modified xylanase cDNA sequence in pNX-Tac is shown in Fig 7.

The specific activity of crude xylanase preparations of pNXD-Tac, pNXS-Tac and pNX-Tac clones were 228, 124 and 672 U/mg of total cellular protein of *E.coli* respectively, measured in 50mM Na-citrate buffer (pH6) and at 50°C (Fig.5). The xylanase synthesised by the clone pNX-Tac was found mainly in the cell pellet, but a small amount of xylanase (about 5%) was released into the culture medium (Table 3). The pNX-Tac xylanase has a temperature optimum at 50°C and retained >80% of the maximum activity from 40°C to 55°C, and 55% of the activity at 60°C (Fig.8). pNX-Tac xylanase has a broad pH range (Fig 9) and is most active at pH5-7.5, 50% at pH8.5 and 20% at pH9.5. The pNX-Tac xylanase has a high activity in the release of reducing sugar from Kraft pulp at 55°C and in tap water (pH was adjusted to pH7 with H_2SO_4, see Fig.8) and remains active in the hydrolysis of xylan from the pulp at 55°C and pH7 for at least 3hr (Fig.10) The pNX-Tac xylanase is able to hydrolyse a significant amount of xylan from Eucalypt and Pine Kraft pulps (Table 4).

Optimisation of growth conditions pNX-Tac clone.

In order to reduce the cost of xylanase production, growth conditions of *E. coli* strain JM83 harbouring pNX-Tac plasmid were investigated. Table 5 shows that on a laboratory scale pNX-Tac clone preferably grows in LBMG medium at 30°C for 24 hr, which produced 2-fold higher xylanase yield than LBS. IPTG is preferably added at the beginning of the cultivation (Table 6).

Xylanase has many industrial applications, such as the pulp and paper industry, food processing, the feed industry and animal production industry. The enzymes produced by these recombinant xylanase clones have no cellulase activity and have the pH and temperature profile (especially the genetically modified xylanase clone, pNX-Tac) fitted to

conditions used for the enzymatic pre-treatment of pulp. Therefore it is believed that the xylanases of the present invention are applicable to the paper and pulp industry.

Sandoz Products Pty Ltd, in the USA, have conducted practical trials using their product, Cartazyme, which is a fungal xylanase (crude), active at 30°C-55°C, pH 3 to 5, and contains 2 xylanases, and have found that a 25-33% reduction in chlorine is possible using 1U xylanase/g pulp. Also the product is brighter than when chemicals alone are used. Another advantage of the xylanase is that it is specific whereas chemicals can attack the cellulose at low lignin contents, leading to reduced fibre strength and other undesirable physical characteristics. It is therefore clear that xylanases could become more important in pulp bleaching and recombinant ones particularly so because of their specificity and high level of expression. In particular, the pNX-Tac xylanase is very active in hydrolysing of xylan from Kraft pulps.

It is also believed that the xylanase of the invention could find a valuable application in the sugar industry and in relation to the treatment of bagasse or other products containing xylan for more efficient disposal as well as for the treatment of feedstock to improve nutritional value. The genetically modified xylanase gene can also be used for modification of rumen bacteria to improve plant fibre utilization by ruminants.

It therefore will be apparent from the foregoing that the invention includes within its scope not only the recombinant xylanase described above but also xylanases derived from other anaerobic fungi as described above which may be prepared by the methods described herein.

The invention also includes within its scope:

(i) DNA sequences derived from these xylanase cDNAs (particularly the sequences in pNPX30, pNXD-Tac, pNXS-Tac and pNX-Tac) and DNA sequences capable of hybridising thereto using a standard nucleic acid hybridisation technique as described in Sambrook et. al. (1989);

(ii) a DNA construct containing a DNA sequence as in (i) operably linked to regulatory regions capable of directing the expression or over-expression of a polypeptide having xylanase activity in a suitable expression host;

(iii) a transformed microbial host capable of the expression or over-expression of the fungal xylanase, harbouring the above mentioned xylanase constructs;

(iv) a polypeptide having xylanase activity produced by expression using a microbial host as in (iii);

(v) amino acid sequence derived from these xylanases, truncations and modifications therefrom, by one skilled in the art.

Plasmid pNX-Tac in E. coli strain JM83 has been deposited at the International Depository ie. Australian Government Analytical Laboratories 17 March 1993 under accession number N93/12211.

In summary the cloning method of the invention is based upon obtaining a large number of recombinant xylanase clones with strong xylanase activity from an anaerobic rumen fungus such as N. patriciarum which were functionally expressed in E. coli. This approach for isolation of fungal xylanase or other plant polysaccharide hydrolases such as cellulases has not been documented prior to this invention. The approach used in this invention is very efficient and requires only a single cloning step to obtain biologically functional recombinant xylanases from an anaerobic fungus. Therefore it takes much less time to obtain biologically functional xylanase clones from a fungal source compared to previous approaches for isolation of plant polysaccharide hydrolases from fungi which are described in the prior art discussed above.

The term "essentially" as used in the appended claims includes within its scope sequences having 70-100% identity to those sequences shown in Figs. 3, 4, 5 and 7.

Table 1

Xylanase activity of recombinant Bacteriophage clones on Xylan - plat assay

	Xylan - clearing zone
λNPX11	L
λNPX12	S
λNPX13	L+++ (9mm)
λNPX14	L
λNPX15	L+
λNPX16	L
λNPX17	S (4mm)
λNPX18	L+
λNPX19	L
λNPX20	L
λNPX21	L+ (7mm)
λNPX22	L
λNPX23	L
λNPX24	L
λNPX25	L+
λNPX26	L++ (8.5mm)
λNPX27	L
λNPX28	L+
λNPX29	L++++ (10.5mm)
λNPX30	L++++ (10.5mm)

L: Large size
S: Small size
Values in parenthesis is diameter of zone.

λNPX11-28 were isolated from initial screening.
λNPX29 and λNPX30 were isolated after further screening of *N.patricianum* cDNA library.

Table 2

Specific activity of the cloned xylanases from N. patriciarum

	Specific activity (U/mg protein)		
	Xylan	CMC*	Crystalline cellulose
pNPX13	41.6	0	0
pNPX21	7.8	0	0
pNPX29	73.5	0	0
pNPX30	113	0	0

* Analysed by CMC plate assay.

Crude enzyme extracts were used for enzyme assay. The reactions were carried out at 40°C in 50 mM Na-citrate, pH6.5, containing 0.25% xylan or 1% Avicel.

Table 3

Specific activity of pNX-Tac xylanase.

Substrate	Cell pellet		Culture supernant
	U/mg protein	U/ml culture	U/ml culture
Xylan	672	726	23
CMC*	0		
Crystalline cellulose (Avicel)	0		

* Analysed on CMC - plate.

E.coli strain JM83 harbouring pNX-Tac plasmid was grown in L-broth at 30°C for 17hrs.

Xylanase activity was measured in 50mM Na-citrate pH6 containing 0.25% Xylan at 50°C and the reducing sugar released was measured as described in the method.

Table 4

Reducing sugar released from Kraft pulp.

Xylanase µl/g dry pulp	mg reducing sugar released/g dry pulp	
	Eucalypt pulp	Pine pulp
0	0	0
10µl	11.9	6.97
100µl	28.9	9.53

The crude xylanase extract from pNX-Tac clone was incubated with 6%(W/V) pulp suspension in tap water at pH 7.0. The hydrolysis was carried out at 52°C for 3 hours.

Table 5

Optimisation of growth conditions of E.coli JM83 harbouring pNX-Tac plasmid.

	IPTG	cell mass at 24hr (g/Litre)	Xylanase yield (Relative activity) 17hr	24hr	30hr
LBS	0.5mM	10		100%	100%
LBSG		11		55%	55%
LBMG	0.1mM	22		168%	168%
	0.5mM	22	151%	200%	200%
	2.5mM	22		190%	190%
LBMHG	0.5mM	20		110%	110%

E.coli strain JM83 harbouring pNX-Tac plasmid was grown in the specified media containing 50µg/ml Amp at 30°C and IPTG was added at the beginning of the cultivation.

Composition of Media, per litre.

LBS: Bacto-tryptone 10g
Bacto-yeast ext. 5g
NaCl 10g
Sucrose (0.4%) 4g
pH 7.2

LBSG: LBS plus
0.4% Glucose

LBMG: Bacto-tryptone 5g
Bacto-yeast ext. 3g
NaCl 0.5g
$Na_2HPO_4.12H_2O$ 15.1g
KH_2PO_4 3g
NH_4Cl 1g
Casamino acids 5g
Sucrose 6g
$CaCl_2$ (100mM) 1ml
$MgSO_4$ (1M) 2ml
Glucose 4g
pH 7.2

LBMHG: LBMG plus
glucose increased to 1% by adding an extra 6g glucose.

Table 6

Optimisation of Induction time of pNX-Tac clone.

IPTG added at	Xylanase yield (relative activity)
0hr	100%
8hr	82%
16hr	40%

E.coli strain JM83 harbouring pNX-Tac plasmid was grown in LBMG containing 50µg/ml Amp and 0.5 mM IPTG at 30°C for 24 hours.

LEGENDS

Figures 1(a), 1(b), 1(c) and 1(d)

Xylan-clearing zones of recombinant bacteriophage clones containing xylanase cDNAs for *N. patriciarum* concerning clones λNPX13, λNPX17, λNPX21 and λNPX26 respectively.

Figure 2

Restriction maps of the highly active xylanase clones isolated from *Neocallimastix patriciarum* cDNA library.

Abbreviations for restriction enzymes:

B, BstXI; E, EcoRI; H, HpaI; K, KpnI; P, PvuII; S, SacI; Sc, ScaI; X, XhoI.

Figure 3

The DNA sequence of pNPX30 xylanase cDNA. The sequence typed in small letters comes from the pBluescript SK vector.

Figure 4

The amino acid sequence of pNPX30 xylanase. The amino acid residues underlined come from the N-terminus of LacZ peptide and encoded by polylinker sequence in the pBluescript SK vector.

Figure 5

The genetically modified constructs of the xylanase cDNA

vector: $pBTac_2$

primers:

PI: 5'-CGGAATTCATG GCT AGC AGA TTA ACC GTC GGT AAT GGT C

PII: 5'-ATACG TAAGC TTAAA CAGTA CCAGT GGAGG TAG

PIII: 5'-CGGAA TTCAT GGCTA GCAAT GGTAA AAAGT TTACT G

PIV: 5'-ATACG TAAGC TTAAC GAGGA GCGGC AGAGG TGG

Abbreviations for restriction enzymes:

B, BstX I; E, EcoR I; H, Hpa I; K, Kpn I; P, Pvu II; S, Sac I; Sc, Sca I; X, Xho I.

Figure 6

pNX-Tac construct

Figure 7

The sequence of the modified xylanase cDNA in pNX-Tac

Figure 8

Effect of incubation temperature on the activity of pNX-Tac xylanase. Xylanase assays were performed in 50 mM Na-citrate (pH7) and 0.25% (w/v) xylan at the various temperatures for 30 min.

Figure 9

Effect of pH on the activity of pNX-Tac xylanase.

Xylanase assays were performed at 50 C in 50 mM Na-citrate (pH5-7) or 25 mM Tris-Cl / 50 mM NaCl (pH7.5-9.5) with 0.25% xylan for 30 min. The pHs of the buffers were measured at room temperature.

Figure 10

Time course of pNX-Tac xylanase activity on eucalypt Kraft pulp. Hydrolysis was carried out at 55°C in tap-water suspended pulp at pH 7.0.

CLAIMS:

1. A method of cloning of xylanase clones from an anaerobic rumen fungus including the steps of:
   (i) cultivation of an anaerobic rumen fungus;
   (ii) isolating total RNA from the culture in step (i);
   (iii) isolating poly A^+ mRNA from the total RNA referred to in step (ii);
   (iv) constructing a cDNA expression library;
   (v) ligating cDNA to a bacteriophage expression vector selected from λZAP, λZAPII or vectors of similar properties;
   (vi) screening of xylanase positive recombinant clones in a culture medium incorporating xylan by detection of xylan hydrolysis; and
   (vii) purifying xylanase positive recombinant clones.
2. A method as claimed in claim 1 wherein the expression vector is λZAPII.
3. A method as claimed in claim 1 wherein the detection of enzyme hydrolysis is carried out using a colour indicator Congo red.
4. A method as claimed in claim 1 wherein after production of xylanase positive clones the cDNA insert in such clones were excised into p Bluescript SK(-) using helper phage.
5. A method as claimed in claim 4 wherein the helper phage is R408 helper phage.
6. Xylanase positive recombinant clones produced by the method of claim 1.
7. Xylanase positive recombinant clones having the following properties:
   (i) production of xylan clearing zones in a culture containing xylanase cDNA derived from N. patriciarum;
   (ii) having activity in hydrolysis of xylan but having no activity in relation to hydrolysis of CMC or crystalline cellulose.

8. Recombinant xylanase clone pNPX21 deposited at the Australian Government Analytical Laboratories on June 22, 1992 under accession number N92/27542.

9. An isolated DNA molecule including a DNA sequenc essentially corresponding to pNPX30 xylanase cDNA as shown in FIG 3 including DNA sequences capable of hybridizing thereto.

10. A polypeptide including amino acid sequence of pNPX30 xylanase essentially as shown in FIG 4.

11. An isolated DNA molecule including a DNA sequence corresponding to pNXD-Tac essentially as shown in FIG 5 including DNA sequences capable of hybridizing thereto.

12. An isolated DNA molecule including a DNA sequence corresponding to pNXS-Tac essentially as shown in FIG 5 including DNA sequences capable of hybridizing thereto.

13. An isolated DNA molecule including a DNA sequence corresponding to pNX-Tac essentially as shown in FIG 5 including DNA sequences capable of hybridizing thereto.

14. Primer PI shown in FIG 5.

15. Primer PII shown in FIG 5.

16. Primer PIII shown in FIG 5.

17. Primer PIV shown in FIG 5.

18. An isolated DNA molecule including a DNA sequence essentially as shown in FIG 7 including DNA sequences capable of hybridizing thereto.

19. Xylanases produced from the recombinant xylanase clones of claim 6.

20. Xylanases produced from the recombinant xylanase clones of claim 7.

21. A DNA construct containing a DNA sequence as claimed in claim 9 operably linked to regulatory regions capable of directing the expression or over-expression of a polypeptide having xylanase activity in a suitable expression host.

22. A DNA construct containing a DNA sequence as claimed in claim 11 operably linked to regulatory regions capable of directing the expression or over-expression of a polypeptide having xylanase activity in a suitable expression host.

23. A DNA construct containing a DNA sequence as claimed in claim 12 operably linked to regulatory regions capable of directing the expression or over-expression of a polypeptide having xylanase activity in a suitable expression host.

24. A DNA construct containing a DNA sequence as claimed in claim 13 operably linked to regulatory regions capable of directing the expression or over-expression of a polypeptide having xylanase activity in a suitable expression host.

25. A DNA construct containing a DNA sequence as claimed in claim 18 operably linked to regulatory regions capable of directing the expression or over-expression of a polypeptide having xylanase activity in a suitable expression host.

26. A transformed microbial host capable of the expression or over expression of fungal xylanase harbouring the xylanase construct of claim 21.

27. A transformed microbial host capable of the expression or over expression of fungal xylanase harbouring the xylanase construct of claim 22.

28. A transformed microbial host capable of the expression or over expression of fungal xylanase harbouring the xylanase construct of claim 23.

29. A transformed microbial host capable of the expression or over expression of fungal xylanase harbouring the xylanase construct of claim 24.

30. A transformed microbial host capable of the expression or over expression of fungal xylanase harbouring the xylanase construct of claim 25.

31. A polypeptide having xylanase activity produc d by expression using a microbial host of claim 26.

32. A polypeptide having xylanase activity produced by expression using a microbial host of claim 27.

33. A polypeptide having xylanase activity produced by expression using a microbial host of claim 28.

34. A polypeptide having xylanase activity produced by expression using a microbial host of claim 29.

35. A polypeptide having xylanase activity produced by expression using a microbial host of claim 30.

36. A polypeptide including amino acid sequences derived from the polypeptide of claim 31 including truncated and modified forms thereof.

37. A polypeptide including amino acid sequences derived from the polypeptide of claim 32 including truncated and modified forms thereof.

38. A polypeptide including amino acid sequences derived from the polypeptide of claim 33 including truncated and modified forms thereof.

39. A polypeptide including amino acid sequences derived from the polypeptide of claim 34 including truncated and modified forms thereof.

40. A polypeptide including amino acid sequences derived from the polypeptide of claim 35 including truncated and modified forms thereof.

41. Plasmid pNX-Tac lodged at the Australian Government Analytical Laboratories on March 17, 1993 under accession number N93/12211.

42. An isolated cDNA molecule which encodes a functional Neocallimastix xylanase.

43. An isolated cDNA molecul which encodes a functional Neocallimastix patriciarum xylanase.

44. A transformed microbial host capable of the expression or over expression of fungal xylanase harbouring the cDNA molecule of claim 42.

45. A transformed microbial host capable of the expression or over expression of fungal xylanase harbouring the cDNA molecule of claim 43.

46. A polypeptide having xylanase activity produced by expression using the microbial host of claim 44.

47. A polypeptide having xylanase activity produced by expression using the microbial host of claim 45.

48. Amino acid sequences derived from the peptide of claim 46 including truncated and modified forms thereof.

49. Amino acid sequences derived from the peptide of claim 47 including truncated and modified forms thereof.

FIB. 1(a)

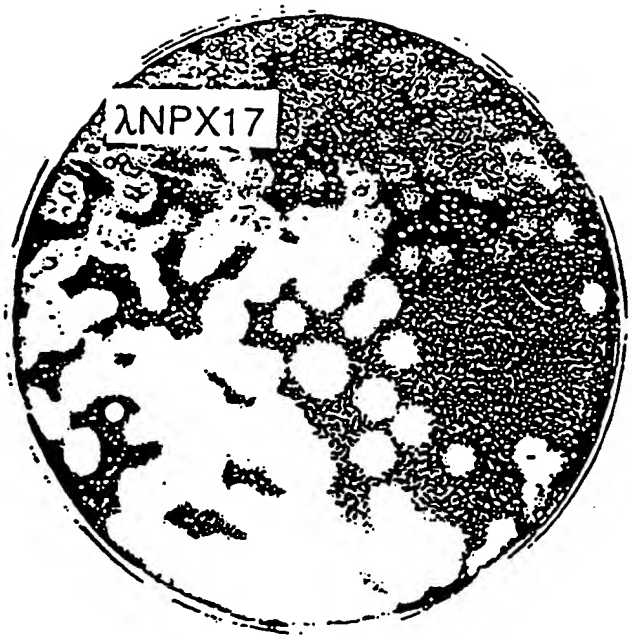

FIG. 1(b)

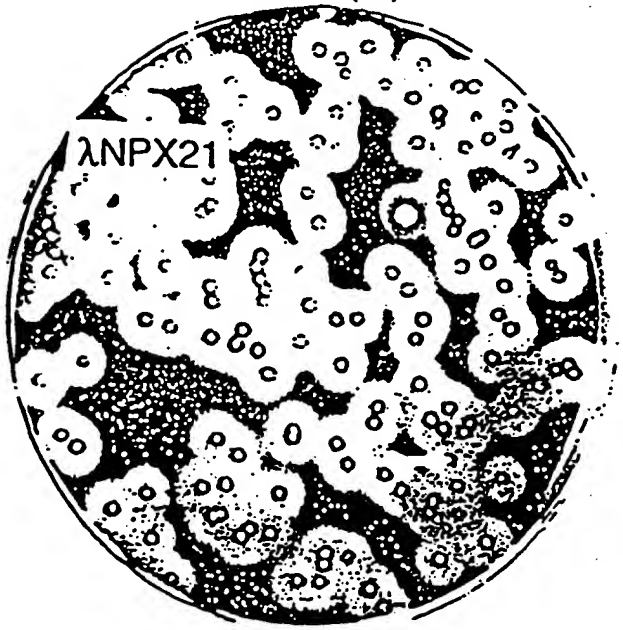

FIG. 1(c)

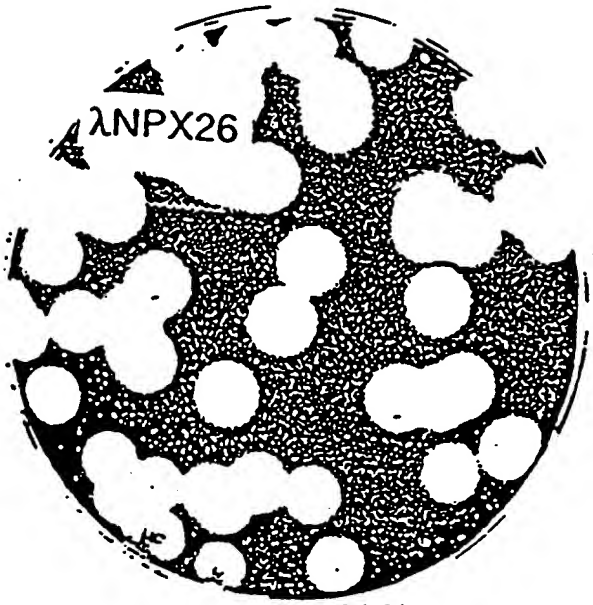

FIG. 1(d)

FIGURE 1

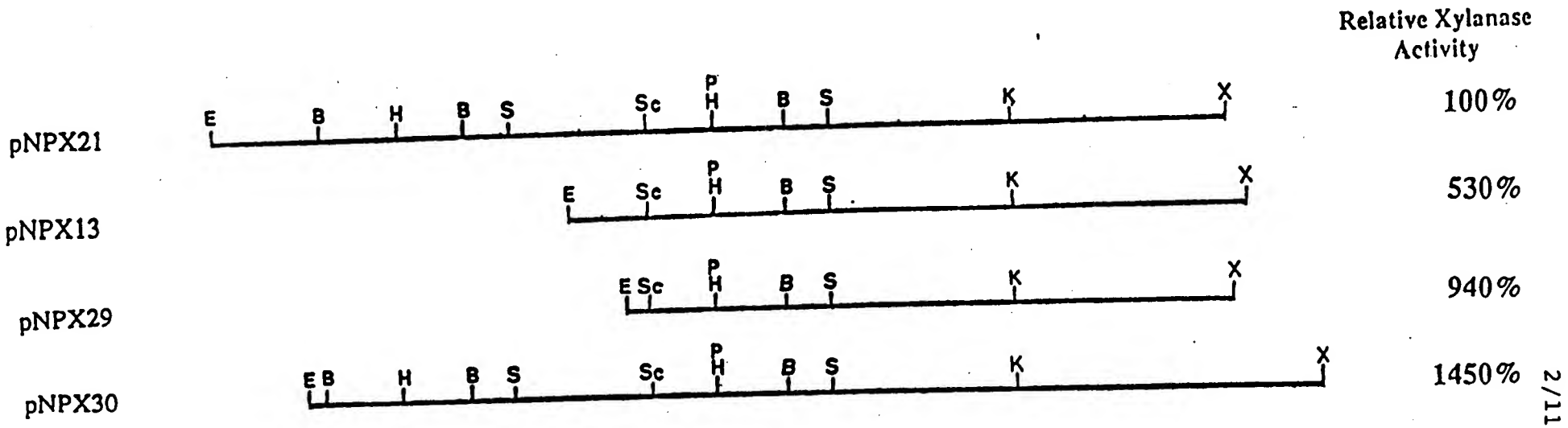
Relative Xylanase Activity
pNPX21
E B H B S Sc PH B S K X
100%
pNPX13
E Sc PH B S K X
530%
pNPX29
E Sc PH B S K X
940%
pNPX30
E B H B S Sc PH B S K X
1450%

FIGURE 2

```
1
atgaccatga  ttacgccaag  ctcgaaatta  accctcacta  aagggaacaa
51
aagctggagc  tccaccgcgg  tggcggccgc  tctagaacta  gtggatcccc
101
cgggctgcag  gaattcggca  cgagGAGAGA  GAGAGAGAGA  GAGAGAGAGA
151
GAGAGAGAGA  GAGCCCAATG  GGGTGGAGGT  GGTGCCTCTG  CTGGTCAAAG
201
ATTAACCGTC  GGTAATGGTC  AAACCCAACA  TAAGGGTGTA  GCTGATGGTT
251
ACAGTTATGA  AATCTGGTTA  GATAACACCG  GTGGTAGTGG  TTCTATGACT
301
CTCGGTAGTG  GTGCAACCTT  CAAGGCTGAA  TGGAATGCAT  CTGTTAACCG
351
TGGTAACTTC  CTTGCCCGTC  GTGGTCTTGA  CTTCGGTTCT  CAAAAGAAGG
401
CAACCGATTA  CAGCTACATT  GGATTGGATT  ATACTGCAAC  TTACAGACAA
451
ACTGGTAGCG  CAAGTGGTAA  CTCCCGTCTC  TGTGTATACG  GTTGGTTCCA
501
AAACCGTGGA  GTTCAAGGTG  TTCCATTGGT  AGAATACTAC  ATCATTGAAG
551
ATTGGGTTGA  CTGGGTTCCA  GATGCACAAG  GTAGAATGGT  AACCATTGAT
601
GGAGCTCAAT  ATAAGATTTT  CCAAATGGAT  CACACTGGTC  CAACTATCAA
651
TGGTGGTAGT  GAAACCTTTA  AGCAATACTT  CAGTGTCCGT  CAACAAAAGA
701
GAACTTCTGG  TCATATTACT  GTCTCAGATC  ACTTTAAGGA  ATGGGCCAAA
751
CAAGGTTGGG  GTATTGGTAA  CCTTTATGAA  GTTGCTTTGA  ACGCCGAAGG
801
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851
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901
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951
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1001
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1051
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1101
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1151
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1201
GGTAACTCCC  GTCTCTGTGT  ATACGGATGG  TTCCAAAACC  GTGGACTTAA
1251
TGGCGTTCCT  TTAGTAGAAT  ACTACATCAT  TGAAGATTGG  GTTGACTGGG
1301
TTCCAGATGC  ACAAGGAAAA  ATGGTAACCA  TTGATGGAGC  TCAATATAAG
1351
ATTTTCCAAA  TGGATCACAC  TGGTCCAACT  ATCAATGGTG  GTAGTGAAAC
```

```
1401
CTTTAAGCAA  TACTTCAGTG  TCCGTCAACA  AAAGAGAACT  TCTGGTCATA
1451
TTACTGTCTC  AGATCACTTT  AAGGAATGGG  CCAAACAAGG  TTGGGGTATT
1501
GGTAACCTTT  ATGAAGTTGC  TTTGAACGCC  GAAGGTTGGC  AAAGTAGTGG
1551
TGTTGCTGAT  GTCACCTTAT  TAGATGTTTA  CACAACTCCA  AAGGGTTCTA
1601
GTCCAGCCAC  CTCTGCCGCT  CCTCGTACTA  CTACCCGTAC  TACTACTCGT
1651
ACCAAGTCTC  TTCCAACCAA  TTACAATAAG  TGTTCTGCTA  GAATTACTGC
1701
TCAAGGTTAC  AAGTGTTGTA  GCGATCCAAA  TTGTGTTGTT  TACTACACTG
1751
ATGAGGATGG  TACCTGGGGT  GTTGAAAACA  ACGACTGGTG  TGGTTGTGGT
1801
GTTGAACAAT  GTTCTTCCAA  GATCACTTCT  CAAGGTTACA  AGTGTTGTAG
1851
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1901
CTGAAAACAA  CGACTGGTGT  GGTTGTGGTT  TCTAAGCAGT  AAAATACTAA
1951
TTAATAAAAA  ATTAAAGAAT  TATGAAAAAT  TTAAATTTAA  AAATTTAAAA
2001
GAATTATGAA  AAATTTAAAT  TTAAAAATTT  AAAAAAAACT  AATTTAGTAA
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2101
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2151
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2201
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2251
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2301
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2351
GATTTAGTAG  AAAAATAAAA  AATTATGAAA  AATTTAAAAT  TTATAAAAAA
2401
TATAAATTTG  TAAAAATTTA  AATGAAAAAT  TTAAAAATAT  AATTTTTAAA
2451
ATAATAAAGA  ATTATGAAAA  ATTAAATATA  AAAAAAAAAA  AAAAAAAAAA
2501
AAA
```

FIGURE 3

M T M I T P S S K L T L T K G N K S W S S T A V A A A L E L V

D P P G C R N S A R G E R E R E R E R E R E R A Q W G G G G A

S A G Q R L T V G N G Q T Q H K G V A D G Y S Y E I W L D N T

G G S G S M T L G S G A T F K A E W N A S V N R G N F L A R R

G L D F G S Q K K A T D Y S Y I G L D Y T A T Y R Q T G S A S

G N S R L C V Y G W F Q N R G V Q G V P L V E Y Y I I E D W V

D W V P D A Q G R M V T I D G A Q Y K I F Q M D H T G P T I N

G G S E T F K Q Y F S V R Q Q K R T S G H I T V S D H F K E W

A K Q G W G I G N L Y E V A L N A E G W Q S S G I A D V T K L

D V Y T T Q K G S N P A P T S T G T V P S S S A G G S T A N G

K K F T V G N G Q N Q H K G V N D G F S Y E I W L D N T G G N

G S M T L G S G A T F K A E W N A A V N R G N F L A R R G L D

F G S Q K K A T D Y D Y I G L D Y A A T Y K Q T A S A S G N S

R L C V Y G W F Q N R G L N G V P L V E Y Y I I E D W V D W V

P D A Q G K M V T I D G A Q Y K I F Q M D H T G P T I N G G S

E T F K Q Y F S V R Q Q K R T S G H I T V S D H F K E W A K Q

G W G I G N L Y E V A L N A E G W Q S S G V A D V T L L D V Y

T T P K G S S P A T S A A P R T T T R T T T R T K S L P T N Y

N K C S A R I T A Q G Y K C C S D P N C V V Y Y T D E D G T W

G V E N N D W C G C G V E Q C S S K I T S Q G Y K C C S D P

N C V V F Y T D D D G K W G A E N N D W C G C G F

FIGURE 4

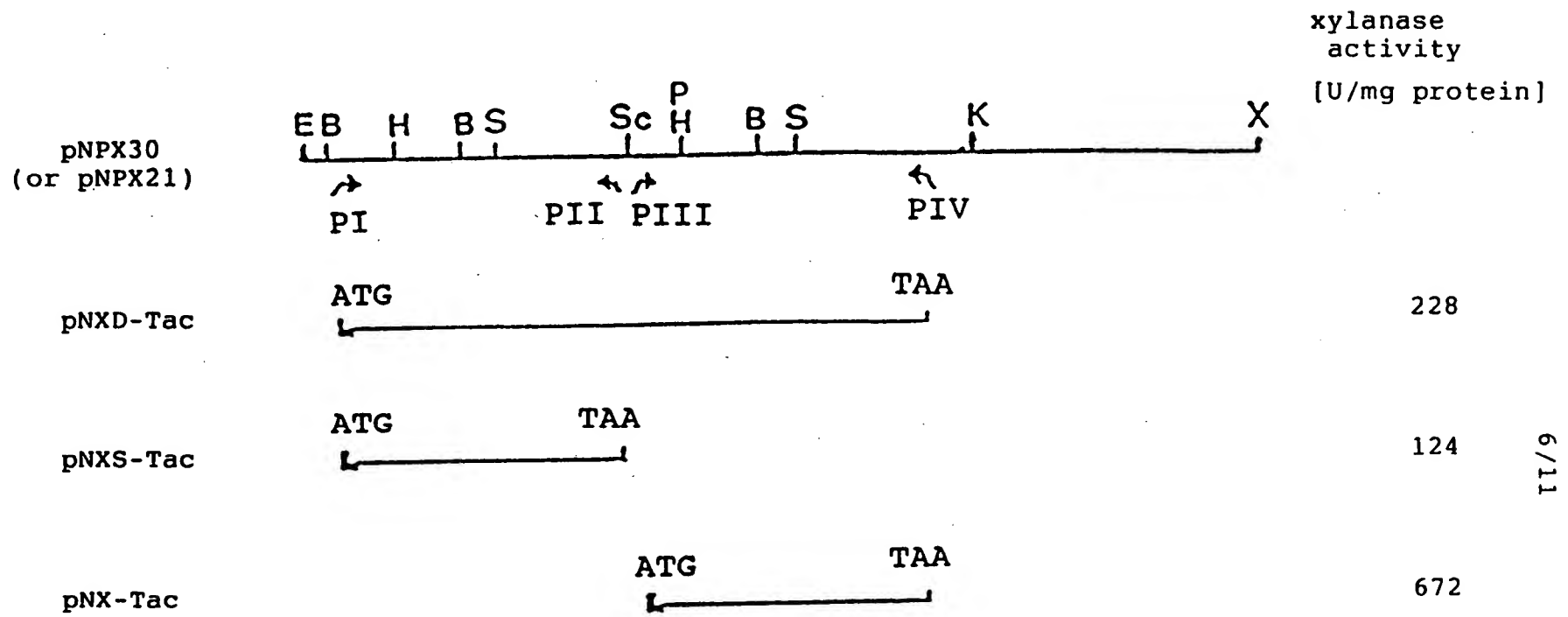
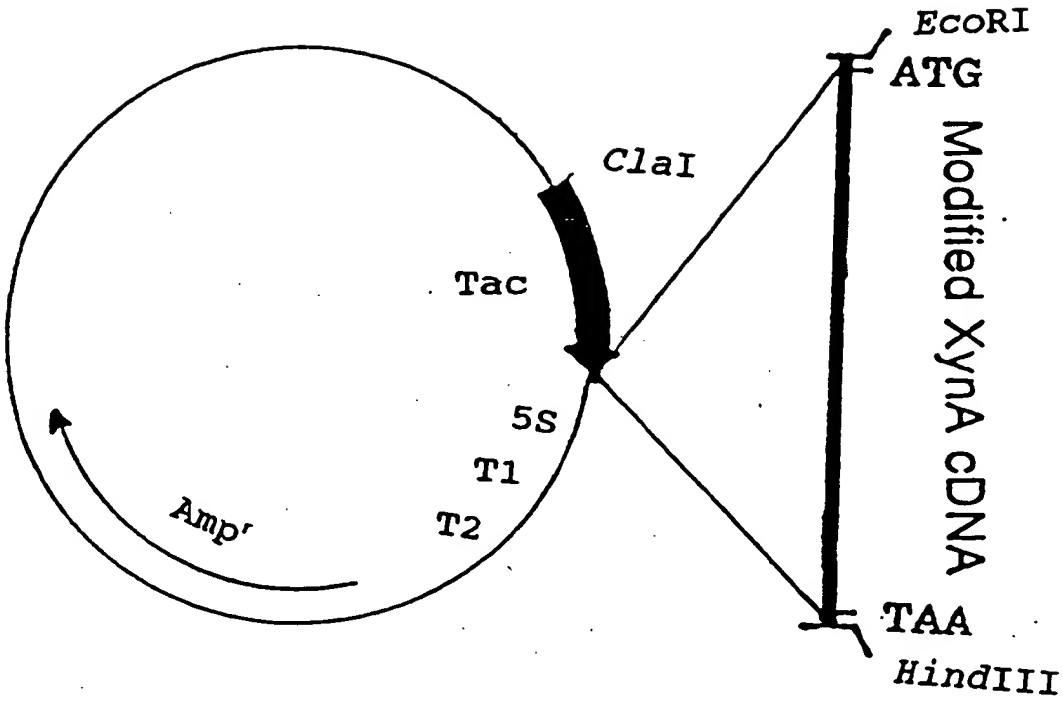


Figure 5



pNX-Tac

Figure 6

```
ATGGCTAGC AATGGTAAAAAGT
M  A  S  N  G  K  K

TTACTGTCGGTAATGGACAAAACCAACATAAGGGTGTCAACGATGGTTTCAGTTATGAAA
F  T  V  G  N  G  Q  N  Q  H  K  G  V  N  D  G  F  S  Y  E

TCTGGTTAGATAACACTGGTGGTAACGGTTCTATGACTCTCGGTAGTGGTGCAACTTTCA
I  W  L  D  N  T  G  G  N  G  S  M  T  L  G  S  G  A  T  F

AGGCTGAATGGAATGCAGCTGTTAACCGTGGTAACTTCCTTGCCCGTCGTGGTCTTGACT
K  A  E  W  N  A  A  V  N  R  G  N  F  L  A  R  R  G  L  D

TCGGTTCTCAAAAGAAGGCAACCGATTACGACTACATTGGATTAGATTATGCTGCTACTT
F  G  S  Q  K  K  A  T  D  Y  D  Y  I  G  L  D  Y  A  A  T

ACAAACAAACTGCCAGTGCAAGTGGTAACTCCCGTCTCTGTGTATACGGATGGTTCCAAA
Y  K  Q  T  A  S  A  S  G  N  S  R  L  C  V  Y  G  W  F  Q

ACCGTGGACTTAATGGCGTTCCTTTAGTAGAATACTACATCATTGAAGATTGGGTTGACT
N  R  G  L  N  G  V  P  L  V  E  Y  Y  I  I  E  D  W  V  D

GGGTTCCAGATGCACAAGGAAAAATGGTAACCATTGATGGAGCTCAATATAAGATTTTCC
W  V  P  D  A  Q  G  K  M  V  T  I  D  G  A  Q  Y  K  I  F

AAATGGATCACACTGGTCCAACTATCAATGGTGGTAGTGAAACCTTTAAGCAATACTTCA
Q  M  D  H  T  G  P  T  I  N  G  G  S  E  T  F  K  Q  Y  F

GTGTCCGTCAACAAAAGAGAACTTCTGGTCATATTACTGTCTCAGATCACTTTAAGGAAT
S  V  R  Q  Q  K  R  T  S  G  H  I  T  V  S  D  H  F  K  E

GGGCCAAACAAGGTTGGGGTATTGGTAACCTTTATGAAGTTGCTTTGAACGCCGAAGGTT
W  A  K  Q  G  W  G  I  G  N  L  Y  E  V  A  L  N  A  E  G

GGCAAAGTAGTGGTGTTGCTGATGTCACCTTATTAGATGTTTACACAACTCCAAAGGGTT
W  Q  S  S  G  V  A  D  V  T  L  L  D  V  Y  T  T  P  K  G

CTAGTCCAGCCACCTCTGCCGCTCCTCGT TAA
S  S  P  A  T  S  A  A  P  R
```

Figure 7

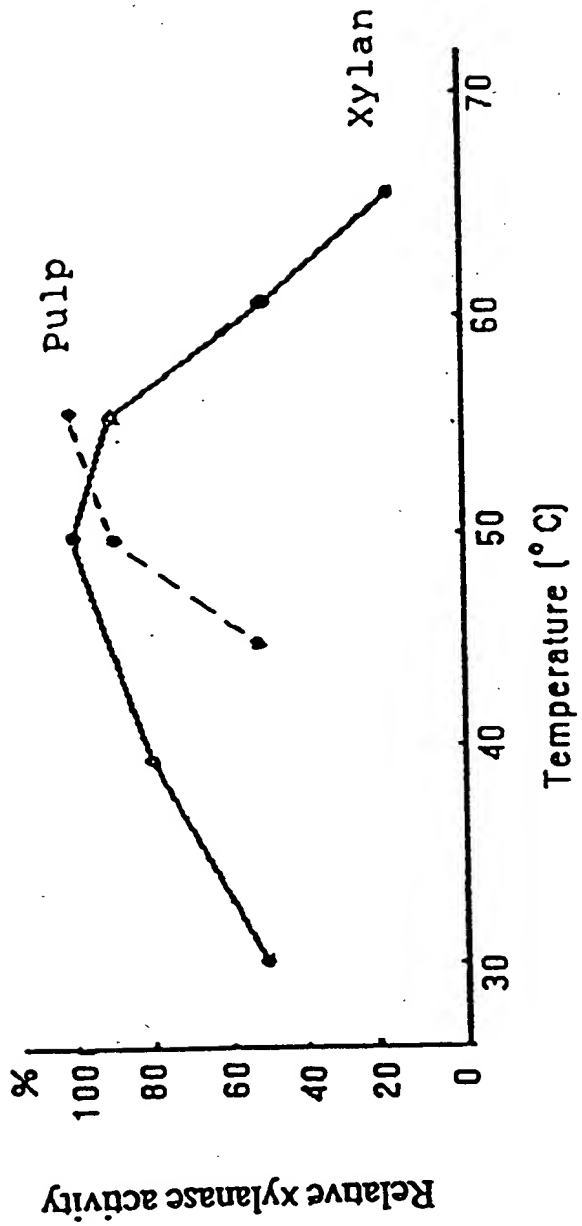

Relative xylanase activity
%
100
80
60
40
20
0
30
40
50
60
70
Temperature (°C)
Pulp
Xylan

FIGURE 8

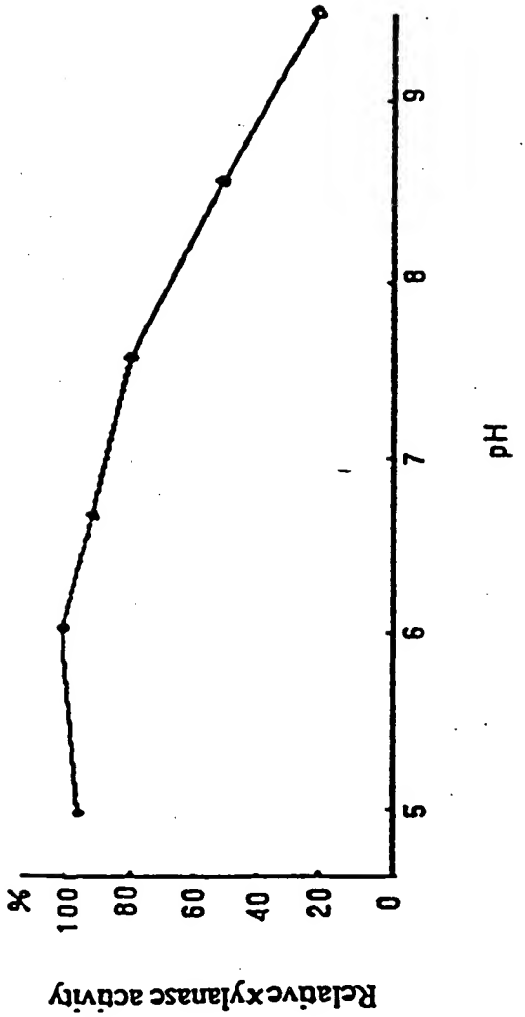


FIGURE 9

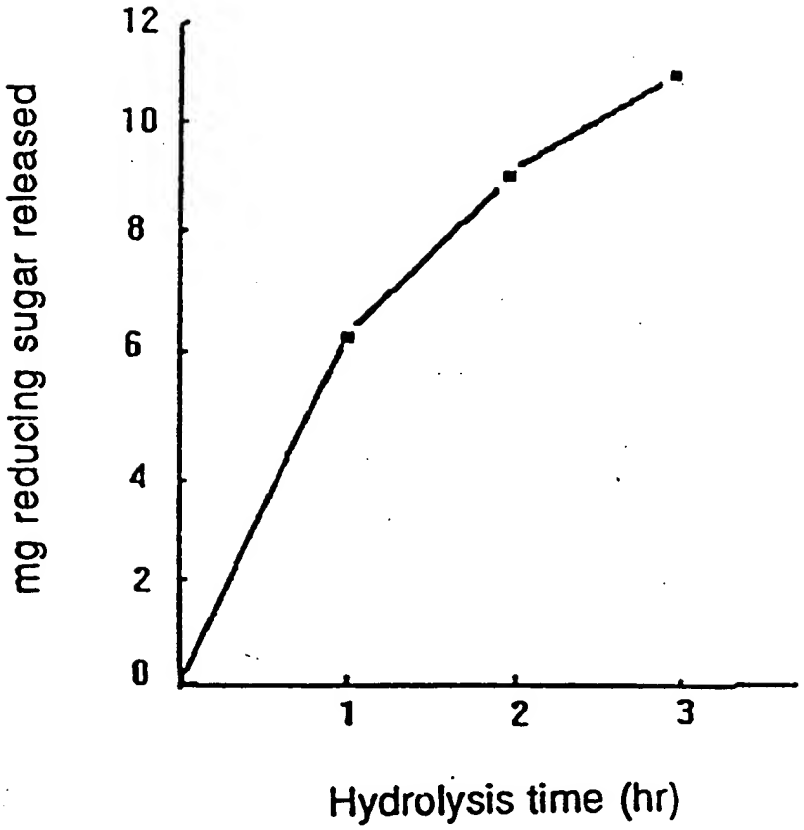


FIGURE 10

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.[5] C12N 9/38, C12N 15/56

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C12N, Derwent Databases: WPAT, Chem Abstracts. K yw rds: as below.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

IPC: AU database C12N 9/38, C12N 15/56

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)

Derwent Databases: WPAT, keywords: xylan:, fung:, C12N/IC; CASM and Biotechnology Abstracts, keywords: xylan:, fung:, recombin:, DNA, gene:, not P/DT

STN Database: oligonucleotide primers as defined in claims 14-17

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X Y	Australian Microbiologist, Vol. 13(3), issued July 1992 by the Australian Society for Microbiology (Parkville, Vic.), G.P. Xue et al, "Expression of a multi-functional cellulolytic cDNA from the rumen fungus Neocallimastix patriciarum in E. coli, page A22	42-47 1,6,7,19
P,Y	Can. J. Microbiol., Vol. 39(1), issued January 1993, J.M. Tamblyn Lee et al, "Cloning of a xylanase gene from the ruminal fungus Neocallimastix patriciarum 27 and its expression in Escherichia coli, pages 134-139	1,6,7,19,42-47
P,Y	Biosci. Biotech. Biochem, Vol. 56(6), issued 1992 by the Society for Bioscience, Biotechnology and Agriculture (Tokyo), K. Ito et al, "Cloning and sequencing of the xynA gene encoding xylanase A of Aspergillus kawachii, pages 906-912	1,6,7,19,42-47

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand th principle or theory underlying the invention

"X" document of particular relevance; the laimed invention cannot be considered novel r cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 1993 (07.09.93)	15 OCT 1993 (15.10.93)

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation f document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
P,Y	Bi sci. Biotech. Biochem, Vol. 56(8), issued 1992 by the Society for Bi science, Biotechnology and Agriculture (Tokyo), K. Ito et al, "Cloning and sequencing of the xynC gene encoding acid xylanase of Aspergillus kawachii, pages 1338-1340	1,6,7,19,42-47
P,Y	FEMS Microbiology Reviews, Vol. 104 (1 and 2), issued January 1993 by the Federation of European Microbiological Societies (Elsevier, Amsterdam), J.A. Thomson, "Molecular biology of xylan degradation", pages 65-82	1
A	AU,A,80668/91 (Unilever PLC) 26 December 1991 (26.12.91)	

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	80668/91	CA	2085232	FI	925730	NL	9001388
		WO	91/19782				

END OF ANNEX